

AD _____

GRANT NUMBER DAMD17-96-1-6052

TITLE: Isolation of Breast Tumor Suppressor Genes from
Chromosome 11p

PRINCIPAL INVESTIGATOR: Doctor Pratima Karnik

CONTRACTING ORGANIZATION: Cleveland Clinic Foundation
Cleveland, OH 44195

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19980116 163

DTIC QUALITY INSPECTED 3

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1997	3. REPORT TYPE AND DATES COVERED Annual (23 Aug 96 - 23 Aug 97)	
4. TITLE AND SUBTITLE Isolation of Breast Tumor Suppressor Genes from Chromosome 11p			5. FUNDING NUMBERS DAMD17-96-1-6052	
6. AUTHOR(S) Dr. Pratima Karnik				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cleveland Clinic Foundation Cleveland OH 44195			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Loss of heterozygosity (LOH) at chromosome 11p15 is commonly observed in breast tumors, indicating the presence of one or more tumor suppressor genes, that map to this chromosomal region. However, positional cloning efforts to identify the target genes on 11p15 have been complicated by the large size of this region and complexity of LOH at 11p15. Furthermore, data correlating 11p LOH in tumors, with their biological and clinical outcome are rare. Here we have precisely defined the location of a breast tumor suppressor gene between the markers D11S1318-D11S1288 (~800kb) within 11p15.5. LOH at this region occurred in ~35-45% of breast tumors analyzed. In addition, we have fine-mapped a second, critical region of LOH that spans the markers D11S1338-D11S1323 (~336kb) at 11p15.5-p15.4, that is lost in approximately 50-60% of breast tumors. We find a striking correlation between the loss of the two 11p loci and the clinical and histopathological features of breast tumors. LOH at 11p15.5 correlated significantly with early events in malignancy and invasiveness. In contrast, the loss of the more proximal 11p15.5-p15.4 locus is highly predictive of aggressive metastatic disease. Thus, two distinct tumor suppressor loci mediate breast tumor progression and metastasis, thereby allowing, a differential prognostic role to be assigned to 11p LOH in the evaluation of breast cancer patients				
14. SUBJECT TERMS Breast Cancer Chromosome 11, Loss of heterozygosity, Tumor suppressor, Lymph node metastasis, Prognostic indicator			15. NUMBER OF PAGES 28	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

P-K For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

PK In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PK In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Pratima Karnik Sep. 10, 1997
PI - Signature Date

TABLE OF CONTENTS:

Front Cover	1
Standard Form (SF) 298, Report Documentation Page	2
Foreward	3
Table of Contents	4
Introduction	5-6
Body	6-13
Results	6-10
Discussion	10-13
Conclusions	13-14
Materials and Methods	14-16
Recommendations in relation to statement of work	16
References	17-21
Table 1	22
Figure Legends	23-24
Appendices	25-28
Figure 1	25
Figure 2	26
Figure 3	27
Figure 4	28

INTRODUCTION:

Breast cancer is both genetically and clinically a heterogeneous and progressive disease. The severity of disease may be determined by the accumulation of alterations in multiple genes that regulate cell growth and proliferation. The inactivation of tumor-suppressor genes, by a 2-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the genetic evolution of breast carcinomas (1). Several chromosome arms, including 1p, 1q, 3p, 11p, 11q, 13q, 16q, 17p, 17q and 18q have been reported to show moderate (20-40%) to high (>50%) frequencies of LOH in breast tumors (1). This implies that multiple tumor suppressor genes are likely involved in the development and progression of breast cancer.

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer (2-16). Several childhood tumors demonstrate LOH for 11p including rhabdomyosarcoma (7), adrenocortical carcinoma (8), hepatoblastoma (9), mesoblastic nephroma (10) and Wilms' tumors (11). Recurrent LOH at 11p is also observed in adult tumors including bladder (12), ovarian (13), lung carcinomas (14), testicular cancers (15), hepatocellular carcinomas (16) and breast carcinomas (2-6) suggesting the presence of one or more critical tumor suppressor gene(s) involved in several malignancies.

Birch et. al. (17) have reported an increased risk for breast cancer among mothers of children with embryonal rhabdomyosarcoma, providing genetic evidence for the apparent high-risk association between these two tumor types. The familial association between breast cancer and rhabdomyosarcoma and the other childhood tumors may well be the consequence of alterations in chromosome 11p15. The ability of a tumor suppressor gene(s) on chromosome 11 to re-establish control on the malignant phenotype has been demonstrated by transfer of a normal human chromosome 11 to the breast cancer cell line MDA-MB-435 (18). However, positional cloning efforts to identify the target genes on

11p15 have been complicated by the large size of this region (~10 Mb) and complexity of LOH at 11p15.

A major challenge in the study of breast cancer is to identify the genes that are involved in the metastatic process that might serve as markers to help detect metastatic cells, to predict the aggressiveness of the disease, and to locate and eradicate metastases. With the goal of identifying the putative tumor suppressor gene(s) on chromosome 11p15, we have refined the minimal regions of LOH in this region, using a high-density marker analysis of 94 informative primary breast tumors and paired normal breast tissue. We have precisely defined and identified two distinct regions of chromosome 11p15.5-p15.4 that are frequently subject to LOH in breast cancer. While the loss of region 1 correlates with early events in malignancy and invasiveness; the loss of region 2, portends a more aggressive disease and regional lymph node metastasis, in the patient. Thus, chromosome 11p15 harbors two distinct tumor suppressor loci that could effectively serve as prognostic indicators in detecting the progression and metastasis of breast cancer.

BODY:

RESULTS:

Refinement of the tumor suppressor loci on chromosome 11p involved in breast cancer:

To identify the smallest common deleted region on chromosome 11p15 in breast tumors, ninety four paired normal/tumor DNAs were assessed for LOH at twelve chromosome 11p15-specific microsatellite loci. These markers encompass the chromosomal sub-regions 11p15.5-11p15.4, estimated to be ~ 8-10 Mb (19) (Fig. 1). The results indicate that the loss of all or part of chromosome 11p is a more common event in human breast cancer than previously appreciated (3,4). LOH occurred in at least one marker on the short arm of chromosome 11 in 56 of 94 (60%) informative tumors. The overall frequency of LOH for each marker varies from 24-60%, with the highest loss seen

at markers D11S1318 (45%) and D11S1338 (60%) (Fig. 1). In addition to the 23% LOH at the D11S988 locus (Fig. 1), there was a high incidence of microsatellite instability (MSI) at this marker as we had described earlier (5). Therefore, the possibility that MSI obscures the accurate determination of LOH at the D11S988 locus in some of these tumors cannot be ruled out.

Tumors 57, 94, 6 and 24 (Genescans- Fig. 2) are illustrative examples of LOH patterns seen on chromosome 11p15 and provide a critical description of the LOH regions. Interstitial deletions, examples of which are seen in tumors 57, 94, 6 and 24, were more commonly observed than loss of the entire chromosomal arm as seen in tumor 7 (Fig. 3). In some cases, example of which is seen at the marker D11S1997 in Tumor 24 (Fig.2), it was observed that the peak for the allele which loses heterozygosity does not change between normal and tumor tissues. Rather, the peak for the other allele increases by several fold in the tumor. Since the surrounding markers show LOH, we believe that this allelic imbalance represents LOH and not gene amplification. The genotypes of the 13 representative breast tumors described in Fig. 3, along with other tumors analyzed (data not shown), serve to identify and refine two distinct regions of LOH on 11p15. Region 1 is encompassed by markers D11S1318 and D11S1288 and is defined by the LOH breakpoints in tumors 57 and 94. Tumor 57 retained heterozygosity for TH and D11S1318, but showed LOH for the markers D11S1288, D11S860, D11S988, HBB and retained heterozygosity for all the remaining proximal markers. Tumor 94 showed LOH at markers TH, D11S1318 and retained heterozygosity at all the proximal markers. Tumors 94 and 57, therefore, refine the LOH Region 1 to a distance of ~800 kb between the markers D11S1318 and D11S1288. This distance was calculated based on the estimation of James et al (19) that $1cR_{900} = 50.2\text{kb}$. Importantly, these results narrow the region containing this tumor suppressor gene from 2 Mb reported earlier (3,4) to ~ 800 kb. Tumors 42, 57 and 94 are examples of tumors that contain interstitial deletions exclusively in region 1 (Fig. 3).

The more centromeric region of LOH (region 2) is defined by breakpoints in the tumors 6 and 24 (Figs.2 and 3). Tumor 6 showed LOH for the markers D11S988, HBB, D11S1760 and D11S1338, but retained heterozygosity at the markers TH, D11S1318, D11S1288, D11S860, D11S1323 and the remaining proximal markers. Tumor 24 was heterozygous for all the distal markers and showed LOH at D11S1323, D11S1331, D11S1997 and D11S866. It is notable that tumors 6 and 24 exhibit LOH at either D11S1338 or D11S1323, while the other locus retains heterozygosity. This clearly indicates that, the region 2 is within the interval that spans the markers D11S1338-D11S1323, a distance of ~ 336 kb, based on the estimate of James et al (19). Tumors 6, 24, 35, 45 and 76 are examples of tumors that harbor interstitial deletions in region 2 (Fig. 3). We have identified integrin linked kinase (p59ILK) as a candidate gene for this locus. p59ILK was previously mapped to the CALC-HBBC region on chromosome 11p15 (36). We have refined the map location of p59ILK and placed it on a yeast artificial chromosome (YAC) 847a12 that is 1440 kb and contains the markers D11S1338 and D11S1323. PCR amplification of DNA from the YAC 847a12 with several different p59ILK primers produced the expected length fragments (data not shown). No products were seen from a BAC DNA specific for the marker D11S1323 or from yeast DNA.

A total of five tumors, examples of which were seen in tumors 7, 20, 26, 30 and 34 (Fig. 3), appeared to have lost both of the regions on the chromosome 11p arm. In tumor 7 (Fig. 3), nine of the markers used, showed LOH. This tumor was non-informative for the markers HBB and D11S866. The probability of three or more allelic losses in the same fragment being caused by independent events is small, and a series of LOH in contiguous markers is more likely due to deletion of the entire segment. In most instances, however, LOH on 11p15 appeared to be interstitial (example tumors 20, 26, 30, 34) and therefore, restricted to relatively small chromosomal regions.

These data attest the presence of two distinct regions of LOH within 11p15.5-15.4. Region 1 lies between markers D11S1318 - D11S1288 (~800 kb) and region 2 lies

between markers D11S1338 - D11S1323 (~336kb). As described in Figure 3, the two regions were lost in different tumors, although in some tumors both of these regions appeared to be lost either due to interstitial deletions or due to the loss of the entire 11p arm.

Correlation between loss of heterozygosity at 11p and clinicopathological features of Breast Tumors:

LOH on chromosome 11 has been widely investigated in a variety of tumors, but because most of the studies have focused on the localization of putative tumor suppressor genes, data about correlations with clinical and histopathologic parameters are rare. To examine the role of 11p LOH in breast cancer and to determine if the two regions are differentially involved in predicting the clinical course of this disease, we correlated our LOH data with the various clinical parameters (Table 1). A correlation was observed between LOH in region 1 and breast tumors containing ductal carcinoma *in situ* (DCIS) synchronous with invasive carcinoma. Fifteen percent (4/26) of ductal tumors with LOH in region 1, contained breast cancer tissues with synchronous DCIS and invasive carcinoma; while none of the tumors with LOH in Region 2 contained a DCIS component ($p = 0.016$). DCIS of the breast is considered a preinvasive stage of breast cancer and may be a precursor of infiltrating breast cancer (20). Therefore, the statistically significant association between LOH in region 1 and such tumors, suggest the involvement of a target gene in this region with early events in malignancy or invasiveness. However, the number of tumors analyzed is small and it remains to be determined if these observations will bear out further study with a larger group of tumors. The statistical analysis showed a significant association between 11p LOH and tumor ploidy. The majority of tumors (16/24) with Region 1 LOH were either diploid or near diploid ($p = < 0.001$). In contrast, the majority of tumors with Region 2 LOH were aneuploid ($p = < 0.001$).

A trend was also observed between LOH at 11p and S-phase fraction (SPF). Fifty four percent of tumors with LOH in Region 2, had a high SPF (>10% of cells in S-phase),

compared with only 32% tumors with LOH in Region 1. However, due to the small number of tumors in each category, statistical significance could not be established. It has been suggested that abnormal ploidy or elevated SPF identifies patients with shorter survival, and worsened disease-free survival, as well as being associated with poor outcome in locoregional control of the disease (21). The association between LOH at region 2 and tumors with high SPF and abnormal ploidy, that we observe, is therefore very relevant.

A striking correlation was observed between loss of region 2 and lymphatic invasion. Importantly, 69% of patients with 11p LOH in Region 2 showed lymphatic invasion, whereas this infiltration was present in only 29% of patients with Region 1 LOH. Thus, tumors that had lost region 2 reveal a significantly higher incidence of metastasis to a regional lymph node(s) ($p = 0.012$) than tumors that had lost Region 1. Tumors that had lost the entire 11p arm, or had lost both regions, showed the clinicopathological features of tumors that had lost Region 2. We also observed the trend that LOH in Region 2 occurs more frequently in higher grade (Grade III) tumors than LOH in Region 1. Thus, LOH at Region 2 may be a late event in mammary tumorigenesis, potentially enabling a clone of previously transformed cells to exhibit greater biological aggressiveness.

DISCUSSION:

We have identified two distinct regions on chromosome 11p15 that are subject to LOH during breast tumor progression and metastasis. The high frequency of somatic loss of genetic information and the striking clinical correlations observed suggests their involvement in the pathogenesis of breast cancer.

We have precisely defined and narrowed the location of the putative tumor suppressor gene in Region 1 from ~ 2 Mb (3,4) to ~800 kb, based on the estimation of physical distances from James et al (19). The critical region appears to extend between the loci D11S1318 and D11S1288 at 11p15.5. Previous studies (3,4) had only been able to

place the putative gene in the larger overlapping area between TH and D11S988 (Fig.4). Although LOH frequencies for this region are consistent (24-45%, this report; 35%, Ref. 3 and 22%, Ref.4), the peak incidence of LOH in this report is highest at D11S1318, ~1Mb distal to the peak at D11S860, reported by Winkvist et al (3) and Negrini et al (4). This discrepancy may reflect the characteristics of the tumor samples analyzed or a difference in interpretation of the corresponding allelic patterns. LOH involving region 1 coincides with regions implicated in the pathogenesis of rhabdomyosarcoma (23), Wilms tumor (WT2) (23), ovarian carcinoma (13), stomach adenocarcinoma (24) and with a region conferring tumor suppressor activity previously identified by genetic complementation experiments (25). Reid et. al. (26) have used a functional assay to localize a 11p15.5 tumor suppressor gene that maps to this region in the G401 cell line.

Inversions and translocations at chromosome band 11p15.5, associated with Beckwith-Wiedemann syndrome and malignant rhabdoid tumors (27) also overlap with both regions of LOH in this study. As illustrated in Fig. 4, several genes that map to this region are subject to allele-specific imprinting (28-32). This raises the possibility that the tumor suppressor gene that maps to Region 1 may be imprinted in a tissue-specific manner. Given the size of our refined LOH region 1, it is possible that a single pleiotropic gene rather than a cluster of genes may play a role in the genesis of different cancers, possibly at different stages of tumor development and progression.

The progression of ductal carcinoma *in situ* (DCIS) to invasive and metastatic breast cancer is often thought to be a consequence of clonal expansions of neoplastic cells with progressively more genetic alterations (20). LOH in Region 1 correlated with tumors that contain ductal carcinoma *in situ* synchronous with invasive carcinoma. This suggests that the loss of a critical gene in this region may be responsible for early events in malignancy or invasiveness. More extensive analysis and isolation of the target gene that maps to this region will be important to establish whether loss or alteration of the same or different genes is involved in each of these cases. p57KIP2 (30) and NAP2 (32) are

potential tumor suppressor candidate genes that map to Region 1. However, single strand conformation analysis and direct sequencing of breast tumors (with LOH) failed to reveal hemizygous mutations in these genes (data not shown).

The second hot spot of LOH (Region 2) in breast tumors is defined by markers D11S1338-D11S1323, which spans a distance of ~ 336 kb, based on the estimation of physical distances from James et al (19). Region 2 is centromeric to the putative WT2 gene (Region 1) and overlaps with LOH regions previously described for non-small cell lung carcinoma (22) and breast cancer (2). Importantly, we have refined this region from 5-10 Mb described earlier (2,22) to ~ 336kb, with the highest incidence of LOH, at the marker D11S1338. Previous studies (2,22), have only analyzed a few markers, sparsely distributed in the region proximal to HBB. Our study, therefore, is the first report of a detailed analysis of markers proximal to HBB which has allowed refinement of the extent of LOH in region 2 in breast cancer.

We observed a significant correlation between loss of heterozygosity at region 2 and the clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. This indicates that LOH in region 2 could serve as a prognostic indicator for identifying patients who are at high risk to develop metastatic disease. The association between 11p LOH and tumor progression and metastasis, that we describe, is analogous to the observations made in other epithelial tumors including breast cancer (6). For example, LOH at 11p correlated with advanced T stage and nodal involvement in Non-small cell lung carcinoma (33) as well as subclonal progression (34), hepatic involvement (13), and poor survival in ovarian and breast carcinomas (3,35). Phillips et. al. (18) have shown that micro-cell mediated transfer of a normal human chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435, had no effect on tumorigenicity in nude mice but suppressed metastasis to the lung and regional lymph nodes. This further supports the observation that chromosome 11 harbors a

metastasis suppressor gene. Integrin-linked kinase (p59ILK) has been mapped to the CALC-HBBC region and was shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents (36). We have refined the map location of p59ILK, and placed this gene between the markers D11S1338 and D11S1323, on the YAC 847a12. Thus, p59ILK is a strong tumor suppressor candidate for region 2.

LOH events at two or more noncontiguous regions of chromosome 11p15 in breast cancer could occur either concurrently through a complex rearrangement of a single chromosome or as independent events. In addition, it is not clear if LOH involving regions 1 and 2 act independently or synergistically in breast tumors. The exclusive association of LOH at region 1 with tumors containing synchronous DCIS and invasive carcinoma, suggests that LOH at the two regions occurs independently and perhaps at different time points during breast tumor progression. Furthermore, we have identified tumors that have lost either region 1 or region 2. This is consistent with the possibility that at least two tumor suppressor genes involved in the progression of breast cancer are located on the chromosome 11p15.5-15.4. These genes may function at distinct stages in the development and progression of breast cancer or, alternatively, novel target genes may be inactivated in different tumors. It is possible, that specific subsets of tumors are defined by the particular set of mutations that they contain, which results in the clinical heterogeneity that is frequently seen in breast cancer.

CONCLUSIONS:

The prognosis in breast cancer is often complicated by the fact that the disease shows a highly variable clinical course (21). Some patients have a rapidly progressive disease with short survival, while others have a long disease-free interval, followed by distant metastases several years after the initial surgery. At present, few markers can reliably predict tumor progression and metastasis in breast cancer. The findings of this paper, allow a differential prognostic role for the two 11p LOH regions, in identifying

patients who are at high risk to develop advanced and metastatic cancer. The identification of tumor suppressor loci on chromosome 11p that are associated with tumor progression and metastasis in breast cancer, should lead to the cloning of the target 11p tumor suppressor genes, and the establishment of the mechanisms whereby they contribute to the evolution of breast cancer.

MATERIALS AND METHODS:

Patient Materials and Preparation of Genomic DNA :

Primary tumor and adjacent normal breast tissue samples were obtained from 94 randomly selected breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). Samples of these tumors and corresponding noninvolved tissue from each patient were collected at the time of surgery, snap-frozen, and transferred to -80°C. Clinical and histopathological features of the tumors described in Table 1 was performed by the Pathology Department at CCF and was revealed only after the LOH study had been completed. An initial cryostat section was stained with H and E stain to determine the proportion of contaminating normal tissue and only DNA purified from specimens thought to be highly enriched in tumor tissue was used for PCR. Generally we use tumor samples that contain less than 40% contamination of normal cells. In cases where LOH is questionable, where possible, regions containing a high proportion of normal tissue were physically removed from the original block by microdissection followed by DNA isolation. These improvements combined with the automatic quantitation of results using the Genescan Analysis have given us a better indication of LOH in tumor samples. Genomic DNA was isolated from normal and tumor tissue samples as described earlier (5) and quantitated by determining the optical densities at 260 and 280 nm.

Microsatellite Polymorphisms and Primers: _

DNA sequences flanking polymorphic microsatellite loci on chromosome 11p15.5 were obtained from the chromosome 11 databases (19) and the Genome Data Base (GDB). Dye labeled (FAM or HEX from Applied Biosystems) primers were either obtained from Research Genetics (Huntsville, Alabama) or synthesized as described earlier (5). Only one primer in each pair was fluorescently labeled so that only one DNA strand was detected on the gel. The physical distances between the polymorphic loci were calculated based on the physical distances of James et al (19). According to their calculation, $1\text{CR}_{9000} = 50.2 \text{ kb}$.

Polymerase Chain Reaction (PCR) and analysis of PCR products using Genescan Software: _

PCR of the DNA sequences was performed as described (5). PCR products were analyzed on Seaquest 6% DNA sequencing gels (Garvin, OK) in 1xTBE buffer in a Model 373A automated fluorescent DNA sequencer (Applied Biosystems) which is a four color detection system. One μl of each PCR reaction was combined with 4 μl formamide and 0.5 μl of a fluorescent size marker (ROX 350, Applied Biosystems). The gel was run for 6 h at 30 W. During electrophoresis, the fluorescence detected in the laser scanning region was collected and stored using the Genescan Collection software (Applied Biosystems). The fluorescent gel data collected during the run was automatically analyzed by the Genescan Analysis program (Applied Biosystems) at the end of each run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height and peak area.

LOH analysis with Genescan:

Fluorescent technology (5) was used to detect and analyze CA repeat sequences. The ratio of alleles was calculated for each normal and tumor sample and then the tumor ratio was divided by the normal ratio, i.e. $T1:T2/N1:N2$, where T1 and N1 are the area

values of the shorter length allele and T2 and N2 are area values of the longer allele product peak for tumor and normal respectively. We assigned a ratio of 0.70 or less to be indicative of loss of heterozygosity on the basis that tumors containing no normal contaminating cells and showing complete allele loss would theoretically give a ratio of 0.0, but because some tumors in this series contained an estimated 30-40% normal stromal cells (interspersed among the tumor cells), complete allele loss in these tumors would give an allele ratio of only 0.70. At least three independent sets of results were used to confirm LOH in each tumor.

Statistical Analysis:

Clinical features of breast tumors are summarized as frequencies and percentages, separately for each region. The Chi-square test was used to compare these features between Regions 1 and 2. All statistical tests were performed using a 5% level of significance.

RECOMMENDATIONS IN RELATION TO THE STATEMENT OF WORK OUTLINED IN THE PROPOSAL:

We have completed Aim 1 of our Statement of Work described in the research proposal.

This work is currently being prepared for publication. The work is progressing on schedule and meets our expectations.

REFERENCES:

1. Callahan, R and Campbell, G.N. Mutations in human breast cancer: an overview. *J. Natl. Cancer Inst.* 81: 1780-1786, 1989.
2. Ali, I.U., Lidereau, R., Theillet, C. and Callahan, R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science* 238: 185-188, 1987.
3. Winqvist, R., Mannermaa, A., Alavaikko, M., Blanco, G., Taskinen, P.J., Kiviniemi, H., Newsham, I. and Cavenee, W. Loss of heterozygosity for chromosome 11 in primary human breast tumors is associated with poor survival after metastasis. *Cancer Res.* 55: 2660-2664, 1995.
4. Negrini, M., Rasio, D., Hampton, G.M., Sabbioni, S., Rattan, S., Carter, S.L., Rosenberg, A.L., Schwartz, G.F., Shiloh, Y., Cavenee, W.K. and Croce, C. Definition and refinement of chromosome 11 regions of loss of heterozygosity in breast cancer: Identification of a new region at 11q23.3. *Cancer Research* 55: 3003-3007, 1995.
5. Karnik, P., Plummer, S., Casey, G., Myles, J., Tubbs, R., Crowe, J. and Williams, B.R.G. Microsatellite instability at a single locus (D11S988) on chromosome 11p15.5 as a late event in mammary tumorigenesis. *Human Mol Genet* 4: 1889-1894, 1995.
6. Takita, K-I., Sato, T., Miyagi, M., Watatani, M., Akiyama, F., Sakamoto, G., Kasumi, F., Abe, R. and Nakamura, Y. Correlation of loss of alleles on the short arms of chromosomes 11 and 17 with metastasis of primary breast cancer to lymph nodes. *Cancer Res.* 52: 3914-3917, 1992.
7. Sait, S.N., Nowak, N.J., Singh-Kahlon, P., Weksberg, R., Squire, J., Shows, T.B., Higgins, M.J. Localization of Beckwith-Wiedemann and Rhabdoid tumor chromosome rearrangements to a defined interval in chromosome band

- 11p15.5. *Genes Chromosomes Cancer* 11: 97-105, 1994.
8. Henry, I., Grandjouan, S., Couillin, P., Barichard, F., Huerre-Jeanpierre, C., Glaser, T., Philip, T., Lenoir, G., Chaussain, J.L., Junien, C. Tumor specific loss of 11p15.5 alleles in del 11p13 Wilms tumor and in familial adrenocortical carcinoma. *Proc. Natl. Acad. Sci.* 86: 3247-3251, 1989.
 9. Koufos, A., Hansen, M.F., Copeland, N.G., Jenkins, N.A., Lampkin, B.C. and Cavenee, W.K. Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. *Nature (Lond)* 316: 330-334, 1985.
 10. Sotel-Avila, D. and Gooch, W.M. III. Neoplasms associated with the Beckwith-Wiedemann Syndrome. *Perspect. Pediatr. Pathol.* 3: 255-272, 1976.
 11. Coppes, M.J., Bonetta, L., Huang, A., Hoban, P., Chilton-MacNeill, S., Campbell, C.E., Weksberg, R., Yeger, H., Reeve, A.E. and Williams, B.R.G. Loss of heterozygosity mapping in Wilms tumor indicates the involvement of three distinct regions and a limited role for nondisjunction or mitotic recombination. *Genes, Chromosomes & Cancer* 5: 326-334, 1992.
 12. Fearon, E.R., Feinberg, A.P., Hamilton, S.H. and Vogelstein, B. Loss of genes on the short arm of chromosome 11 in bladder cancer. *Nature* 318: 377-380, 1985.
 13. Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visetin, M.C. and Biocchi, M. Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. *Br. J. Cancer* 66: 1030-1036, 1992.
 14. Bepler, G. and Garcia-Blanco, M.A. Three tumor-suppressor regions on chromosome 11p identified by high-resolution deletion mapping in human non-small cell lung cancer. *Proc. Natl. Acad. Sci. USA* 91: 5513-5517, 1994.
 15. Lothe, R.A., Fossa, S.D., Stenwig, A.E., Nakamura, Y., White, R., Borresen, A.L., Brogger, A. Loss of 3p or 11p alleles is associated with testicular cancer tumors. *Genomics* 5: 134-138, 1989.

16. Wang, H.P. and Rogler, C.E. Deletions in human chromosome arms 11p and 13q in primary hepatocellular carcinomas. *Cytogenet. Cell Genet.* 48: 72-78, 1988.
17. Birch, J.M., Hartley, A.L., Marsden, H.B., Harris, M. and Swindell, R. Excess risk of breast cancer in the mothers of children with soft tissue sarcomas. *Br. J. Cancer* 49: 325-331, 1984.
18. Phillips, K.K., Welch, D.R., Miele, M.E., Lee, J-H., Wei, L.L. and Weissman, B.E. Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11. *Cancer Res.* 56: 1222-1227, 1996.
19. James, M.R., Richard, C.W., III, Schott, J.J., Yousry, C., Clark, K., Bell, J., Terwilliger, J.D., Hazan, J., Dubay, C., Vignal, A., Agrapart, M., Imai, T., Nakamura, Y., Polymeropoulos, M., Weissenbach, J., Cox, D.R. and Lathrop, G.M. A radiation hybrid map of 506 STS markers spanning human chromosome 11. *Nat. Genet.*, 8: 70-76, 1994.
20. Fujii, H., Marsh, C., Cairns, P., Sidransky, D. and Gabrielson, E. Genetic progression, histological grade, and allelic loss in ductal carcinoma in situ of the breast. *Cancer Res.* 56: 1493-1497, 1995.
21. Merkel, E. and McGuire, W.L. Ploidy, proliferative activity and prognosis. DNA flow-cytometry of solid tumors. *Cancer* 65: 1194-1205, 1990.
22. Tran, Y.K. and Newsham, I.F. High-density marker analysis of 11p15.5 in Non-small cell lung carcinomas reveals allelic deletion of one shared and one distinct region when compared to breast carcinomas. *Cancer Research* 56: 2916-2921, 1996.
23. Besnard-Guerin, C., Newsham, I., Winquist, R. and Cavenee, W.K. A common loss of heterozygosity in Wilms tumor and embryonal rhabdomyosarcoma distal to the D11S988 locus on chromosome 11p15.5. *Hum. Genet.* 97: 163-170, 1996

24. Baffa, R., Negrini, M., Mandes, B., Rugge, M., Ranzani, G.N., Hirohashi, S. and Croce, C.M. Loss of heterozygosity for chromosome 11 in adenocarcinoma of the stomach. *Cancer Res.* 56: 268-272, 1996.
25. Koi, M., Johnson, L.A., Kalikin, L.M., Little, P.F.R., Nakamura, Y. and Feinberg, A.P. Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11. *Science* 260: 361-364, 1993.
26. Reid, L.H., West, A., Gioeli, D.G., Phillips, K.K., Kelleher, K.F., Araujo, D., Stanbridge, E.J., Dowdy, S.F., Gerhard, D.S., Weissman, B.E. Localization of a tumor suppressor gene in 11p15.5 using the G401 Wilms tumor assay *Hum. Mol. Genet.* 5: 239-247, 1996.
27. Hoovers, J.M.N., Kalikin, L.M., Johnson, L.A., Alders, M., Redeker, B., Law, D.J., Blik, J., Steenman, M., Benedict, M., Wiegant, J., Lengauer, C., Taillon-Miller, P., Schlessinger, D., Edwards, M.C., Elledge, S.J., Ivens, A., Westerveld, A., Little, P., Mannens, M. and Feinberg, A.P. Multiple genetic loci within 11p15 defined by Beckwith-Wiedemann syndrome rearrangement breakpoints and subchromosomal transferable fragments. *Proc. Natl. Acad. Sci.* 92: 12456-12460. 1995.
28. Zhang, Y., Shields, T., Crenshaw, T., Hao, Y., Moulton, T. and Tycko, B. Imprinting of human H19: allele-specific CpG methylation, loss of the active allele in Wilms tumor, and potential for somatic allele switching. *Am. J. Hum. Genet.* 53: 113-124, 1993.
29. DeChiara, T.M., Robertson, E.J., Efstratiadis, A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* : 64, 849-859, 1991.
30. Matsuoka, S., Thomson, J.S., Edwards, M.C., Bartletta, J.M., Grundy, P., Kalikin, L.M., Harper, J.W., Elledge, S.J., Feinberg, A.P. Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57KIP2, on chromosome 11p15. *Proc. Natl. Acad. Sci.* 93: 3026-3030, 1996.

31. Lee, M.P., Hu, R.-J., Johnson, L.A. and Feinberg, A.P. Human KVLQT1 gene shows tissue-specific imprinting and encompasses Beckwith-Wiedemann syndrome chromosomal rearrangements. *Nature Genetics*:15, 181-185, 1997.
32. Hu, R.J., Lee, M., Johnson, L. and Feinberg, A. A novel human homologue of yeast nucleosome assembly protein, 65 kb centromeric to the p57Kip2 gene, is biallelically expressed in fetal and adult tissues. *Human Mol. Genet.* 5: 1743-1748, 1996.
33. Fong, K.M., Zimmerman, P.V. and Smith, P.J. Correlation of loss of heterozygosity at 11p with tumor progression and survival in non small cell lung cancer. *Genes, Chromosomes and Cancer* 10: 183-189, 1994.
34. Vandamme, B., Lissens, W., Amfo, K., De Sutter, P., Bourgain, C., Vamos, E., De Greve, J. Deletion of chromosome 11p13-1p15.5 sequences in invasive human ovarian cancer is a subclonal progression factor. *Cancer Res.* 52: 6646-6652, 1992.
35. Eccles, D.M., Gruber, L., Stewart, M., Steel, C.M., Leonard, R.C. Allele loss on chromosome 11p is associated with poor survival in ovarian cancer. *Dis Markers* 10: 95-99. 1992.
36. Hannigan, G.E., Bayani, J., Weksberg, R., Beatty, B., Pandita, A., Dedhar, S. and Squire, J. Mapping of the gene encoding the integrin-linked kinase, ILK, to human chromosome 11p15.5-p15.4 *Genomics* : 177-179, 1997.

Table 1**11p LOH and clinico-pathologic features of sporadic breast tumors:**

Clinical Features Variable/Responses	LOH in Region 1		LOH in Region 2		p-value
	N	%	N	%	
Ductal					
Yes	26	86.7	35	87.5	0.92
No	4	13.3	5	12.5	
If Ductal					
In situ and invasive	4	15.4	0	0.0	0.016*
Invasive	22	84.6	35	100.0	
Lobular					
Yes	4	13.3	5	12.5	0.92
No	26	86.7	35	87.5	
If lobular					
In situ and invasive	1	25.0	0	0.0	0.24
Invasive	3	75.0	5	100.0	
Ploidy					
Diploid or near diploid	16	66.7	4	16.0	<0.001*
Aneuploid	8	33.3	21	84.0	
% S-phase cells					
≤ 10%	17	68.0	12	46.2	0.12
> 10%	8	32.0	14	53.9	
ER/PR status					
ER+/PR+	3	23.1	8	50.0	0.23
ER+/PR-	7	53.9	4	25.0	
ER-/PR-	3	23.1	4	25.0	
Grade					
I-II	10	33.3	9	26.5	0.16
II-III	15	50.0	12	35.3	
III	5	16.7	13	38.2	
Lymphatic Invasion					
Yes	4	28.6	20	69.0	0.012*
No	10	71.4	9	31.0	

*statistically significant (p<0.05)

N=number of tumors in each category

FIGURE LEGENDS:**Fig. 1:**

Representation of 11p15.5-15.4 and approximate position of the microsatellite repeats (19). Histogram shows the percentage of LOH for each of these microsatellites in the informative breast samples studied.

Fig. 2:

LOH studies of normal (N) and tumor (T) breast cancer pairs. Genescans of samples 57 (D11S1318, D11S1288, D11S860, D11S988, HBB, D11S1760), 94 (TH, D11S1318, D11S1288, D11S860, D11S988, HBB), 6 (D11S860, D11S988, HBB, D11S1760, D11S1338, D11S1323) and 24 (D11S1760, D11S1338, D11S1323, D11S1338, D11S1997, D11S866) are shown. Arrows represent allelic loss. LOH represents samples that exhibit loss of heterozygosity and was calculated as described in the text.

Fig. 3:**Genotypes of thirteen representative tumors and the smallest regions of shared LOH in sporadic breast carcinoma:**

Tumor numbers are listed across the top, with the markers analyzed to the left. Open circles represent informative samples, with no LOH; filled circles represent informative samples with LOH; and stippled circles represent noninformative (homozygous) samples. The maximum area of LOH is boxed for each LOH region in each tumor. The bars to the right represent the extent of the proposed common regions of LOH (Regions 1 and 2). Tumors that exhibit LOH at Region 1 only, Regions 1 and 2 and Region 2 only are grouped together.

Fig. 4:

Schematic representation of regions on chromosome 11p15.5-15.4 harboring potential tumor suppressor and/or disease loci described in the present study and by other groups in breast cancer (2-5), Wilms tumor (23), Non small cell lung carcinoma (22), Rhabdomyosarcoma (23), Beckwith-Wiedemann syndrome (27) and in stomach adenocarcinoma(24).

APPENDICES

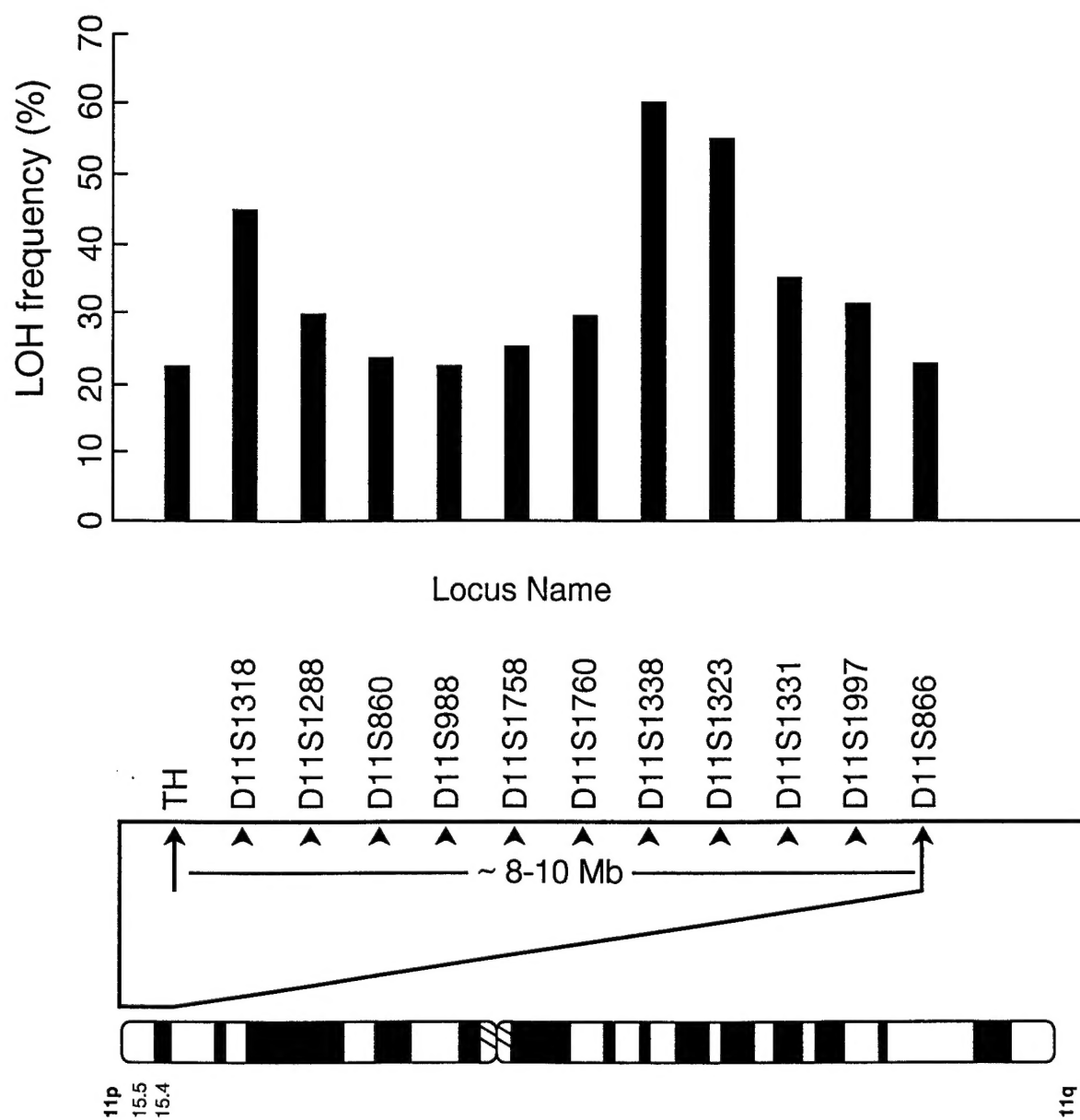


Figure 1

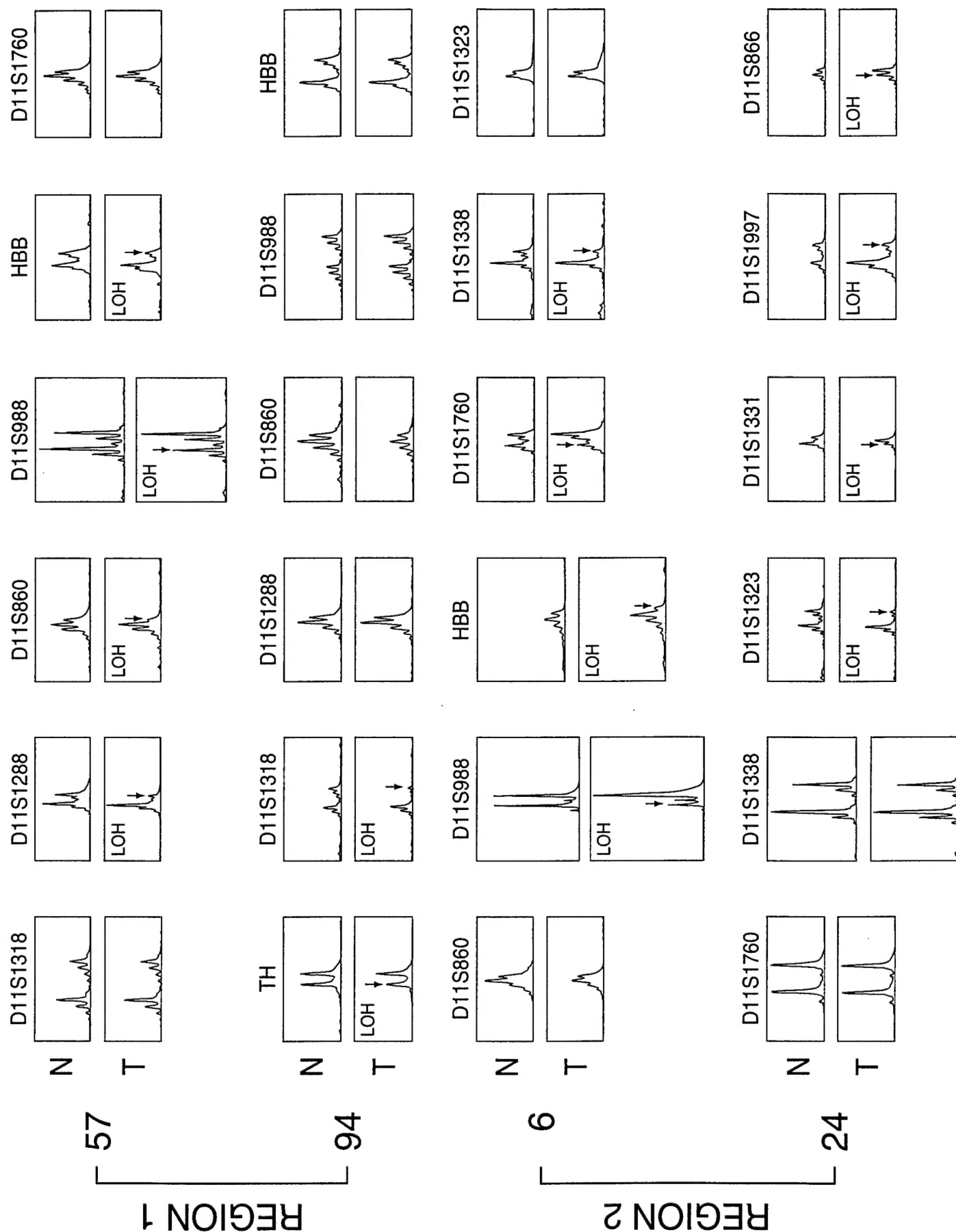




Figure 3

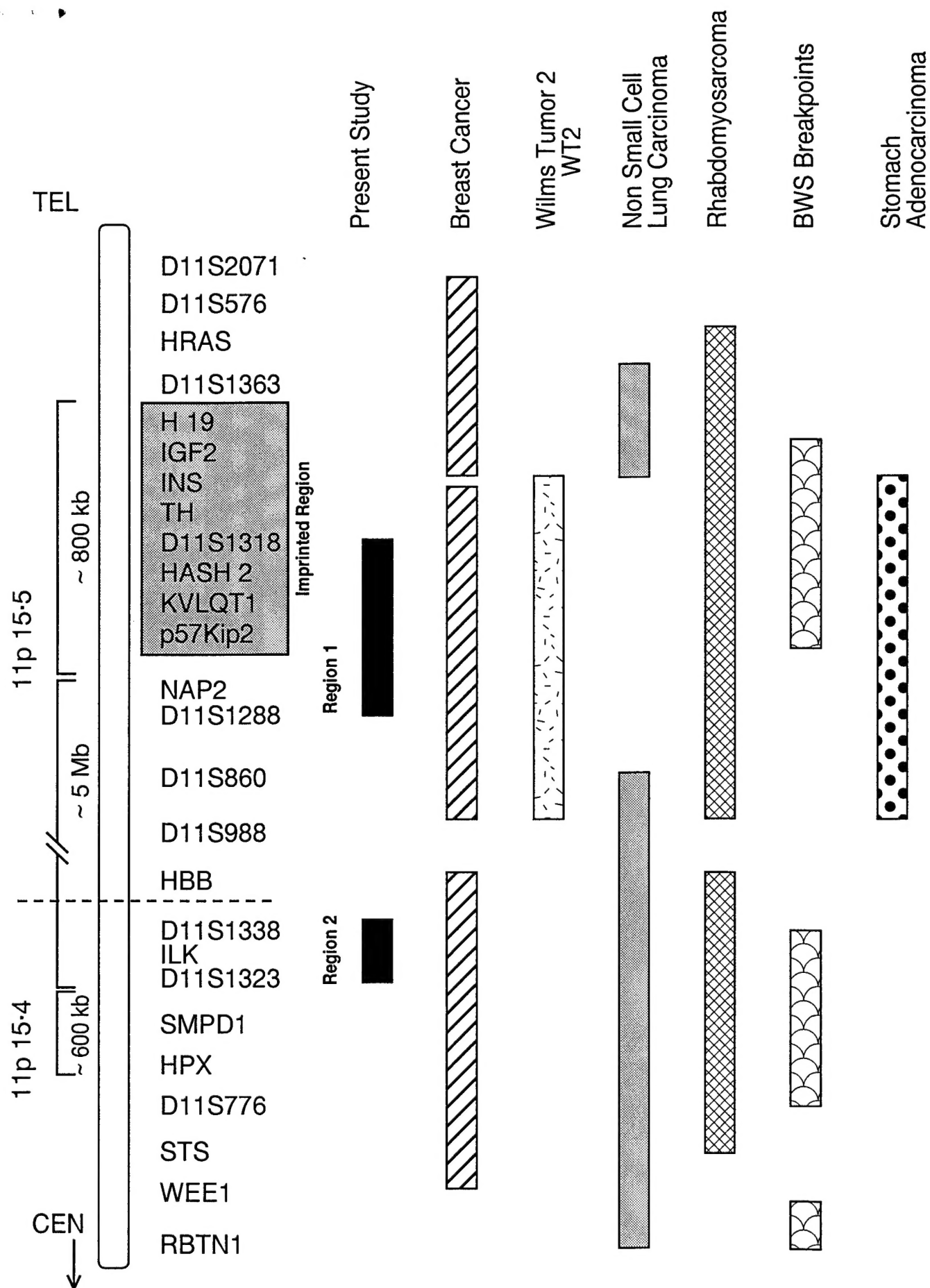


Figure 4